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14. ABSTRACT
BRCA2-Rad51 interaction is required for the Rad51-related DNA repair pathway. Thus, inhibition of their interaction is expected to sensitize tumor cells to certain DNA damaging agents. A panel of 14080 natural compounds from the Chinese National Center for Drug Screening has been partially screened using a yeast two-hybrid system utilizing specific Rad51/BRCA2 constructs. Growth of the Rad51/BRCA2 yeast strain in different media lead us to the selection of 20 candidate inhibitors for BRAC2-Rad51 interaction. We are actually testing if these compounds sensitize breast cancer cell lines to cisplatin. One of the compounds at the concentrations attainable increases the toxicity of cisplatin in a BRCA1 deficient breast cancer cell line.

15. SUBJECT TERMS
Breast Cancer

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A. SUMMARY OF THE PROJECT

BACKGROUND

BRCA2 directly interacts with Rad51 promoting Rad51 directed DNA repair. Repair of interstrand crosslinks induced by interstrand crosslinking agents, involves the Rad51 related homologous recombinational repair pathway (HRR) (1). The homologous recombinational repair process requires the assembly of multienzymatic complexes visualized immunocytochemically as Rad51 nuclear foci. These complexes include the Rad51 paralogs family members such as (Rad51, Rad52, Rad54, Rad51B, Rad51C, Rad51D,Xrcc2and Xrcc3) and the breast cancer associated proteins,BRCA1 and BRCA2. Defective cell lines in each of the above mentioned proteins present similar phenotypes: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents, mild sensitivity to gamma rays and attenuated Rad51 focus formation after exposure to ionizing radiation (2,3). Our innovative contribution will be to find natural compounds to sensitize tumor cells to chemotherapeutic agents by inhibiting BRCA2/Rad51 interaction.

RATIONALE

BRCA2 is central to HRR repair through BRC-mediated Rad51 interactions required for the assembly of DNA damage-induced RAD51 foci. Inhibition of their interaction would sensitize tumor cells to DNA crosslinking agents. Therefore our drug discovery program focus on the identification of compounds capable of competitively block the interaction BRAC2 and Rad51 (3).

OBJECTIVE

Find natural compounds that will inhibit BRCA2-Rad51 interaction in order to inhibit the homologous recombinational process and consequently sensitize breast tumor cells to therapeutic agents.

SELECTION of BRCA2-Rad51 CANDIDATE INHIBITORS

We have cloned the BRCA2 domain that interacts with Rad51 and full-length Rad51 and utilize these cDNA sequences to screen the panel of 15,000 natural compounds from the Chinese National Center for Drug Screening system using a yeast two hybrid system. We determine that 18 compounds displaying selective inhibition of BRCA2-Rad51 interaction had IC_{50} values less than 10μg/ml.

BIOLOGICAL EVALUATION of CANDIDATE INHIBITORS

The biological activity of selected compounds using the two yeast hybrid system was tested in a sporadic human breast cancer cell line panel (expressing wild type BRCA2) using the NCI sulfhorodamine B assay(5). All tests were performed using sublethal doses of the selected compounds, in combination with cisplatin 0-40 μ of cisplatin. Drug interactions (antagonism, additive, or synergism) were assessed as described (6). The ability of to alter homologous recombinational repair will be examined immunocytochemically looking at changes on cisplatin-induced Rad51 foci in the presence of selected compounds. Disruption of Rad51-BRCA2 interaction will be confirmed by cross immunoprecipitation followed by western blot analysis using specific antibodies

B. ORIGINAL STATEMENT OF WORK

Task # 1 Construction of the BRCA2-Rad51 Yeast 2 Hybrid System
STATUS: COMPLETED (first year)

Task #2 Screening of candidate compounds to inhibit BRCA2-RAD51 interaction
STATUS: COMPLETED (first and second year)

Task #3 Biological Evaluation of candidate inhibitors of BRCA2-Rad51 interaction
STATUS: IN PROGRESS (second year)

C. BODY of REPORT

C.1. Task # 1 Construction of the BRCA2-Rad51 Yeast 2 Hybrid System

The BRCA2 domain which interacts with Rad51, was cloned from a human cDNA library using specific primers for human wild type BRCA2 (Accession Number NM_000059). The cDNA sequence corresponding to BRCA2 3196-3991 was amplified by PCR and subcloned into the pBTM116 vector in frame to LexA (BRCA2-LexA hereafter)(6).

The full length Rad51 was cloned from a human cDNA library using specific primers for human wild type Rad51 (Accession Number NM_002875). The Rad51 full cDNA was amplified by PCR and subcloned into the plasmid PACT2 vector in frame to GAL4-TA (GALA4-TA-Rad51 hereafter)(7).

We confirm the proper orientation of the inserts and sequence by agarose electrophoresis after restriction endonuclease digestion and sequencing respectively.

The vectors were transformed either alone or together into the yeast strain L40 (*MAT***a** *trp1 leu2 his3 LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ)* as shown in **Table 1**.The L40 strain can't growth in medium lacking the amino acids leu, trp and his. After transformation the yeast were grown in selective medium (SD) lacking the amino acids leu, trp and his. BRCA2-LexA or GALA4-TA-Rad51 transformed yeast were able to growth in medium containing His but lacking Trp or Leu respectively. Only yeast transformed with both vectors in which the fusion proteins interact was able to growth in SD media lacking the three amino acids (Leu, Trp and His)(7).

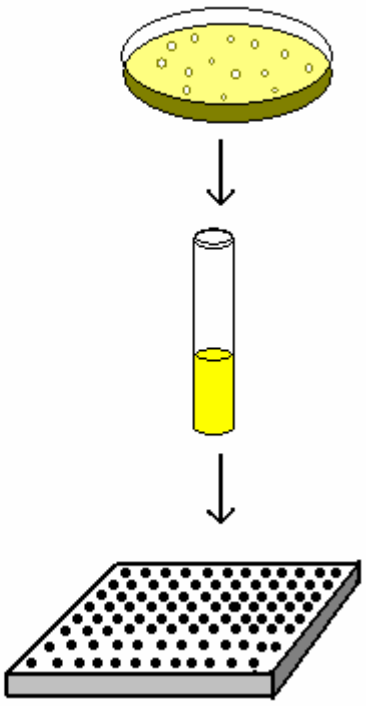
Table 1

Yeast Transformation	Growth	
pBTM116-BRCA2 vector	Negative	**Growth Tested in SD-LTH agar plates:** yeast nitrogen base without amino acids without Leu, Trp and His, supplemented with dextrose and 3-AT*
pPACT2-Rad51 vector	Negative	
pBTM116-BRCA2 + pPACT2 vectors	Negative	
pBTM116 + pPACT2-Rad51 vectors	Negative	
pBTM116-BRCA2+ pPACT2-Rad51 vectors	Positive	

*__3-AT__, 3-Amino-Triazol is used to inhibit the basal expression of *His3* to avoid the growth of false positive yeast colonies.

C.2. Task #2 <u>Screening of candidate compounds to inhibit BRCA2-RAD51 Interaction.</u>

C.2.1.Primary Screening strategy



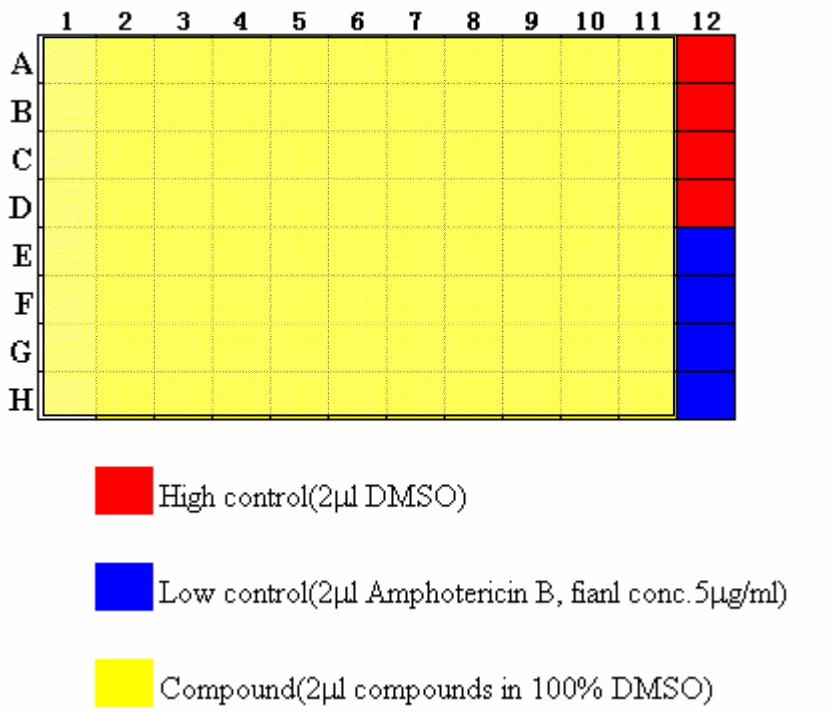
After transfection with the pBTM116-BRCA2 and the pPACT2-Rad51 vectors, the L40 yeast were plated on SD/-Leu/-Trp/-His/ (1mM 3-AT) to select for colonies, in which BRCA2 and RAD51 interact.

The clones were inoculated and grown to the mid-log phase in SD/-Leu/-Trp/-His/ (1mM 3-AT).

The cultures were diluted about 40 folds and transferred to each well (200 µl) of the 96 well plate, containing has 2 µl of a compound (1mg/ml) in 100% DMSO.

The plates were incubated overnight then read at 600nm in a microplatereader to determine the grow status.

96 well Plate format



(DMSO is the vehicle used to dissolve the compounds and Amphotericin B is an anti-yeast agent).

Using this format, the activity of 80 compounds can be obtained from one 96 well plate.
If a given compound inhibits the interaction between BRCA2 and Rad51, the growth of the yeast is inhibited due to the lack of His in the medium. The growth inhibition is calculated respect to the growth in the presence of vehicle (2 µl DMSO).

C.2.2.Primary screening results

The primary screening was carried out as described in **C.2.1.** with 14,080 compounds, and the final concentration of each compound was 10 µg/ml in SD/-Leu/-Trp/-His/ (1mM 3-AT) media.
The percentage of inhibition and the distribution rate of inhibition obtained are shown in **Table 2**.

Table 2. Inhibition rate

% of Inhibition	≥80	≥85	≥90	≥95
Hits	**259**	171	114	73
Hit rate	**1.84%**	1.21%	0.81%	0.52%

C.2.3.Secondary screening strategy

The compounds (Hits) that showed ≥80% of inhibition of BRCA2 and RAD51 interaction during the primary screening (259 in total, **Table 2**) were chosen for further analyses. Sister cultures were grown in the presence of 10 μg/ml of each compound plus SD/-Leu/-Trp/-His/ (1mM 3-AT) or SD/-Leu/-Trp. Inhibition of growth in the first medium (without Leu, Trp and His) indicated specific inhibition of BRCA2-Rad51 interaction while inhibition of growth in the second medium (without Leu and Trp) indicated toxicity.

From the 259 compounds, 130 have been already tested using the secondary screening, 20 of which showed selective growth inhibition in medium lacking Leu, Trp and His respect to the medium lacking Leu and Trp (**Table 3**).

Table 3. **Inhibition rate comparison**.

	SD/-LTH	SD/-LT(+His)
	Inhibition%	
1	91.2	23.3
2	80.6	36.0
3	88.6	44.1
4	84.8	52.1
5	80.1	19.5
6	80.1	33.8
7	80.8	-7.0
8	80.1	37.4
9	96.8	40.1
10	80.1	35.6
11	89.0	-3.0
12	91.7	27.6
13	96.8	30.9
14	89.6	-3.1
15	91.7	30.2
16	97.7	45.2
17	80.4	35.0
18	91.8	18.9
19	86.7	9.5
20	94.3	38.5

C.2.4 IC_{50} value of inhibitor candidates

IC_{50} determination was made for the 20 compounds that showed selectivity in the secondary screening. From a serial dilution of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 μg/ml, 18 compounds were found to have IC_{50} values less than 10 μg/ml in SD/-Leu/-Trp/-His/ (1mM 3-AT) (**Table 3**).

Table 3. IC_{50} value.

	SD/-LTH	SD/-LT(+his)	SD/-LTH
	%Inhibition		IC_{50}(µg/ml)
1	91.2	23.3	6.91
2	80.6	36.0	8.67
3	88.6	44.1	7.29
4	84.8	52.1	4.21
5	80.1	19.5	5.51
6	80.1	33.8	5.34
7	**80.8**	**-7.0**	**3.33**
8	80.1	37.4	2.06
9	96.8	40.1	2.88
10	80.1	35.6	>10
11	89.0	-3.0	>10
12	91.7	27.6	2.69
13	96.8	30.9	1.07
14	**89.6**	**-3.1**	**3.77**
15	91.7	30.2	2.46
16	97.7	45.2	2.53
17	80.4	35.0	4.27
18	91.8	18.9	8.09
19	**86.7**	**9.5**	**6.84**
20	94.3	38.5	6.90

In bold are indicated the best candidates to be tested to sensitize breast cancer cells to cisplatin as described in **TASK #3**

D.3 Task #3 Biological Evaluation of candidate inhibitors of BRCA2-Rad51 interaction

Biological effect of the candidate BRCA2-Rad51 inhibitory-compounds were tested in a panel of four well characterized sporadic breast cancer cell lines purchased from ATCC. The cell lines, MCF-7, T47D, Z75R, CAMA1 and NMDA231 express wild type BRCA2 and Rad51. We also include in the panel two commercially available BRCA2 deficient cell lines, CAPAN-1 as a negative control. The biological effect was determined using the NCI-SRB assay (7). Briefly, exponentially growing cells were plated in 96 well plates (2000 cells/well) in RPMI media containing 10% FBS and treated next day with cisplatin 0-40µM alone or in combination with 5µM of Compound 19. Five days later, the cells were fixed with cold TCA (10% final concentration), washed with water and incubated for half hour with the SRB reagent. After extensive washes with 0.1% acetic acid stained cells were lysed with 10mM Tris. The OD was measured using an Elisa-Plate reader at 450nM. The inhibitory dose ten, fifty and ninety (IC_{10}, IC_{50} and IC_{90}) were obtained by logarithmic interpolation: $y = a\ Ln(x) + b$ were y is OD and x cisplatin concentration. The IC_{10}, IC_{50} and IC_{90} are defined as the x value corresponding to y equal 10, 50 or 90 respectively.
From the compound tested so far (five) only compound 19 (see Task# 2) was soluble in DMSO at a

concentration of DMSO which was not toxic to the cells (0.05%DMSO in culture media). The results obtained are summarized in **Table 4**

TABLE 4

		CAPAN-1	MCF-7	T47D	ZR75	CAMA1	NDA-MB231
Cisplatin	IC_{50}	0.87±0.1	1.78 ±0.07	23.36±2.06	3.83±0.82	5.23±1.17	3.12±0.034
	IC_{10}	15.02±039	10.17±0.75	ND	ND	ND	ND
Alone	IC_{90}	0.048±0.01	0.30±0	0.84±0.13	0.16±0.049	0.28±0.84	0.10±0.021
Cisplatin	IC_{50}	0.78±0.41	1.73±0.11	19.47±3.43	3.2±0.2	4.43±0.06	**2.25±0.27***
+ 5µM	IC_{10}	10.25±1.09	9.00±0.3	ND	ND	ND	ND
Comp19	IC_{90}	0.083±0.07	0.3±0.056	1.06±0.27	0.16±0.02	0.28±0.02	0.07±0.025

The values represent the mean value of 3 independent experiments for each cell line plus minus the standard deviation. ND indicates that the cisplatin concentration range was not high enough to reach the IC_{10} value. Statistically significant difference between the mean values was assessed using the paired t-test for means. There was not significant differences between the sensitivity of the breast cancer cells to cisplatin alone vs. cisplatin compound 19 (5µM). The* indicate significant differences between means ($p< 0.05$).

Form the cell line panel tested, the only cell line sensitized to cisplatin by compound 19 was MDA-MB 231. As expected, the sensitivity to cisplatin of CAPAN-1 cells was not affected by compound 19. Interestingly MDA-MB231 cells are deficient for BRCA1 expression (8). Because it has been shown that BRCA1is necessary for Rad51 related DNA repair process, it is possible that MNDA231 cells are more sensitive to the disruption of BRCA2-Rad51 interaction after DNA repair in the absence of functional BRAC1. We predict that compound 19 will sensitize MCF-7,T47D, ZR75 and CAMA1 cells at higher concentration (> 5µM), but the solubility of the compound is limiting. We are actually waiting for our collaborator Dr Jia Li at the Chinese National Center for Drug Screening to provide us with information regarding the structure of compound 19 to improve the solubility of the compound to be able to proceed with further biological and biochemical studies to confirm its effect on BRCA2-Rad51 interaction. We are also expecting to receive soon from him the additional 15 inhibitor candidates to test their effect on cisplatin sensitivity in our cell line panel.

D. KEY RESEARCH ACCOMPLISHMENTS

- We find 20 candidate inhibitors of the BRCA2-Rad51 interaction after screening of a library of 20,000 natural compounds interacting using a yeast 2 hybrid system.

- On of this compounds sensitize BRCA1 deficient cells to cisplatin.

E. REPORTABLE OUTCOMES There are not reportable outcomes associated with this research yet.

F. CONCLUSIONS. Form the cell line panel tested, the only cell line sensitized to cisplatin by compound 19 was MDA-MB 231. As expected, the sensitivity to cisplatin of CAPAN-1 cells was not affected by compound 19. Interestingly MDA-MB231 cells are deficient for BRCA1 expression (8). Because it has been shown that BRCA1is necessary for Rad51 related DNA repair process, it is possible that MDA-MB231 cells are more sensitive to the disruption of BRCA2-Rad51 interaction after cisplatin-induced DNA damage in the absence of functional BRAC1. We predict that compound 19 will sensitize MCF-7, T47D, ZR75 and CAMA1 cells at higher concentration (> 5μM), but the solubility of the compound is limiting. We are actually waiting for our collaborator Dr Jia Li at the Chinese National Center for Drug Screening to provide us with information regarding the structure of compound 19 to improve the solubility of the compound to be able to proceed with further biological and biochemical studies to confirm its effect on BRCA2-Rad51 interaction. We are also expecting to receive soon from him the additional 15 inhibitor candidates to test their effect on cisplatin sensitivity in our cell line panel.

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